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Epigenome-wide association study of DNA methylation and adult asthma in the Agricultural Lung Health Study

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Distinct methylation signals are found in non-atopic and atopic asthma. Most are related to gene expression and are replicated in asthma-relevant tissues, confirming the value of blood DNA methylation for identifying novel genes linked in asthma pathogenesis. <https://bit.ly/2VnbJg3>

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ABSTRACT Epigenome-wide studies of methylation in children support a role for epigenetic mechanisms in asthma; however, studies in adults are rare and few have examined non-atopic asthma. We conducted the largest epigenome-wide association study (EWAS) of blood DNA methylation in adults in relation to non-atopic and atopic asthma.

We measured DNA methylation in blood using the Illumina MethylationEPIC array among 2286 participants in a case-control study of current adult asthma nested within a United States agricultural cohort. Atopy was defined by serum specific immunoglobulin E (IgE). Participants were categorised as atopy without asthma (n=185), non-atopic asthma (n=673), atopic asthma (n=271), or a reference group of neither atopy nor asthma (n=1157). Analyses were conducted using logistic regression.

No associations were observed with atopy without asthma. Numerous cytosine-phosphate-guanine (CpG) sites were differentially methylated in non-atopic asthma (eight at family-wise error rate (FWER) $p < 9 \times 10^{-8}$, 524 at false discovery rate (FDR) less than 0.05) and implicated 382 novel genes. More CpG sites were identified in atopic asthma (181 at FWER, 1086 at FDR) and implicated 569 novel genes. 104 FDR CpG sites overlapped. 35% of CpG sites in non-atopic asthma and 91% in atopic asthma replicated in studies of whole blood, eosinophils, airway epithelium, or nasal epithelium. Implicated genes were enriched in pathways related to the nervous system or inflammation.

We identified numerous, distinct differentially methylated CpG sites in non-atopic and atopic asthma. Many CpG sites from blood replicated in asthma-relevant tissues. These circulating biomarkers reflect risk and sequelae of disease, as well as implicate novel genes associated with non-atopic and atopic asthma.

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Introduction

Asthma is a heterogeneous, chronic disease of the airways that affects over 300 million people worldwide [1]. Although asthma has a substantial familial component, only a small portion of the variation in disease risk and heritability is explained despite large genome-wide association studies (GWAS) [2, 3]. Epigenetic mechanisms may contribute to the unexplained variation and the best-studied epigenetic modification is DNA methylation.

Most epigenome-wide association studies (EWAS) of methylation in relation to asthma have been conducted in children [4]; however, asthma risk factors and pathogenetic mechanisms vary between children and adults [3]. Compared to childhood asthma, a higher proportion of adult asthma is non-atopic and pathogenesis of asthma may differ by atopy status. The few published EWAS in adults have fewer than 80 asthma cases, measured DNA methylation using the older Illumina Infinium 27K BeadChip or 450K BeadChip, or did not stratify by atopy status.

Using DNA from blood, we conducted the largest EWAS of DNA methylation and adult asthma to date, using the more comprehensive Illumina Infinium MethylationEPIC BeadChip, which assesses methylation at over 850 K cytosine-phosphate-guanine (CpG) sites. To better elucidate the pathogenesis of asthma, we stratified by atopy, defined objectively by specific immunoglobulin E (IgE). We assessed the potential functional impact of the differentially methylated CpG sites through enrichment of functional genomic features, pathway analyses, associations with gene expression and identification of druggable targets. We replicated our differentially methylated CpG sites in blood using existing studies with methylation assessed in whole blood, purified eosinophils, nasal epithelium, or bronchial airway epithelium.

Methods

Study population

Participants were enrolled in the Agricultural Lung Health Study (ALHS), a case-control study of adult current asthma nested within the Agricultural Health Study (AHS). The AHS is a cohort of farmers and their spouses from Iowa and North Carolina. Details of the AHS and ALHS have been described previously [5, 6]. The ALHS enrolled 3301 participants in the period 2009–2013.

Based on responses to an AHS questionnaire administered in the period 2005–2010 (data version P3REL201209.00), the ALHS enrolled asthma cases using three definitions: 1) self-reported current diagnosed asthma without any self-reported diagnosis of chronic obstructive pulmonary disease (COPD) or emphysema ($n=876$); 2) potential undiagnosed asthma identified by self-report of current asthma symptoms or use of asthma medication without COPD or emphysema diagnosis among never smokers or light, former smokers (≤ 10 pack-years) ($n=309$); or 3) current self-reported diagnosed asthma with COPD or emphysema diagnosis among never smokers or light, former smokers (≤ 10 pack-years) ($n=38$). Controls ($n=2078$) were randomly selected from the AHS participants without the above criteria. A full description of the methods can be found in the supplementary material.

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All participants provided informed consent and the Institutional Review Board at the National Institutes of Health approved this study.

Stratification by atopy

Atopy was determined by a positive blood IgE test (based on $\text{IgE} \geq 0.70 \text{ IU}\cdot\text{mL}^{-1}$) [7] to at least one of 10 common antigens: Bermuda grass, ragweed, Timothy grass, mountain cedar, *Alternaria*, dust mite, cat dander, milk, egg and wheat. IgE was measured at ImmuneTech (Foster City, CA, USA) using the Luminex platform (Luminex Corp., Austin, TX, USA). Asthma case-control status was stratified by atopy to categorise individuals into one of four mutually exclusive groups: atopy alone (individuals with atopy but without asthma), non-atopic asthma (individuals with asthma but no atopy), atopic asthma (individuals with asthma and atopy), or non-cases (individuals with neither asthma nor atopy).

DNA methylation and quality control

The DNA was bisulfite converted using the EZ-96 DNA Methylation kit (Zymo Research Corp., Irvine, CA, USA). Methylation was assessed for 2391 ALHS participants using the Infinium MethylationEPIC BeadChip, following Illumina's protocol.

Sample level quality control excluded 102 participants with either 1) $>5\%$ of CpG sites with detection p-values greater than 1.0×10^{-10} or 2) intensity values less than three standard deviations below the mean bisulfite control intensities. In addition, one participant was excluded due to a sex mismatch. CpG sites were removed if $>5\%$ of samples had detection p-values greater than 1.0×10^{-10} ($n=31\,533$). Background correction and dye-bias correction were done using "Relic" in ENmix [8, 9]. Data were normalised using inter-array (quantile) normalisation [8]. Probe-type bias adjustment was conducted using the "Rcp" function in ENmix [10]. We corrected for batch effect (*i.e.* plate effect) using "ComBat" in sva [11]. We used "gaphunter" in minfi to trim extreme methylation outliers at individual CpG sites (threshold=0.3, outCutoff=0.0025) [12]. We analysed 817 235 autosomal CpG sites.

Among the 2286 participants with methylation data passing quality control and with complete information on smoking history, there were 185 with atopy alone, 673 with non-atopic asthma, 271 with atopic asthma and 1157 non-cases (supplementary figure E1).

Cell type proportions

Details of the peripheral blood smears have been described previously [13] and were available in 1894 participants. Monocytes, lymphocytes, neutrophils and eosinophils were counted in smears without platelet clumping and $<20\%$ smudged cells. Cell type proportions were calculated from counts for 1658 participants. For individuals without cell counts, cell type proportions were estimated from methylation data using the Houseman method [14] with the REINUS *et al.* [15] reference panel.

Identification of differentially methylated CpG sites

Epigenome-wide analyses were conducted using logistic regression separately for three outcomes: atopy alone, non-atopic asthma and atopic asthma. Non-cases were the referent group. Untransformed β methylation values were used as the predictor. All analyses were adjusted for age (continuous), sex, body mass index (BMI) (continuous), smoking status (never, former, or current), pack-years of smoking (number of packs smoked per day \times years of smoking, continuous), state of residence (North Carolina or Iowa) and four cell type proportions (monocyte, lymphocyte, neutrophil and eosinophil). As farmers were less likely to be enrolled in the busy autumn harvest season, we also adjusted for autumn enrolment (yes/no). Significance was assessed using a family-wise error rate (FWER) of $p < 9 \times 10^{-8}$ [16], as well as a Benjamini–Hochberg false discovery rate (FDR) of less than 0.05 [17].

Several sensitivity analyses were conducted. To evaluate possible influences of asthma-related genotypes on the associations, we calculated a weighted polygenic risk score for asthma using the results from a large genome-wide meta-analysis of asthma [2]. Single nucleotide polymorphisms (SNPs) with $p < 5 \times 10^{-8}$ were clumped using PLINK ($r^2=0.5$) to identify independent loci within $\pm 250 \text{ kb}$ [18]. Methylation analyses were repeated adjusting for the polygenic risk score of asthma. We also repeated the analyses adjusting for current farming status (*i.e.* currently farming crops, working with farm animals, or working with pesticides). Analyses were conducted using the seven Houseman estimated cell types (monocyte, NK, B-cell, CD4^+ , CD8^+ , neutrophil and eosinophil) instead of the four measured cell types. As we used a stricter threshold to classify atopy than some previous studies, we conducted methylation analyses after re-stratifying asthma case-control status with an atopy cut-off of $\geq 0.35 \text{ IU}\cdot\text{mL}^{-1}$. Analyses were conducted in R version 3.4.0 (www.r-project.org).

Examination of polymorphic probes and additional filtering

We excluded cross-reactive probes [19], probes with SNPs at the extension base (minor allele frequency >1%) [20] and “ch” probes from downstream analyses (*i.e.* enrichment of functional genomic features, pathway analyses, expression quantitative trait methylation, druggable targets and replication look-ups). We visually inspected distributions of significant CpG sites for departure from unimodality.

Enrichment of functional genomic features

We localised CpG sites to four genomic features: CpG islands, CpG island shores, promoters and transcription factor binding sites. We evaluated enrichment or depletion for these features among FDR-significant CpG sites using a two-sided Fisher’s exact test and applied a Bonferroni threshold ($p < 0.05/4 = 0.0125$). Enrichment of transcription factor motifs was identified using eFORGE TF [21]. We used eFORGE version 2.0 to identify tissue-specific and cell type-specific enrichment in blood, lung and fetal lung for the following functionally relevant genomic features: DNase I hypersensitive sites, 15 chromatin states and five histone marks [21].

Pathway analyses

We conducted pathway analyses using the “gsameth” function in the missMethyl package in R [22], which accounts for the differing number of probes per gene. Pathways within the Kyoto Encyclopedia of Genes and Genomes (KEGG) gene sets from the Gene Set Enrichment Analysis Molecular Signatures Database (GSEA MSigDB) [23–25] were evaluated. We reported pathways with a nominal *p*-value of less than 0.05.

Expression quantitative trait methylation (eQTM) analyses

We evaluated association of significant CpG sites with expression of nearby genes (*cis*-eQTMs). We found no studies with both gene expression and blood methylation based on the EPIC array. Therefore, we evaluated eQTMs for the significant CpG sites present on the 450K array. Illumina 450K methylation and gene expression data from RNA-seq were available in blood samples from 3075 adults in the Biobank-based Integrative Omics Study (BIOS) consortium [26]. We evaluated *cis*-eQTMs using expression transcripts within ± 250 kb of each significant CpG site. Significance was assessed at FDR less than 0.05.

Search for druggable targets among novel asthma genes

A previous systematic literature review [27] identified asthma-related genes from EWAS or a large asthma GWAS and we updated their search to incorporate more recent studies. Genes not previously associated with asthma were annotated to the ChEMBL database version 25 (released on January 02, 2019) to identify approved or in development drugs that target novel genes implicated by our significant CpG sites [28].

Replication of findings in blood, eosinophils, nasal and bronchial epithelium

We looked up our FDR significant CpG sites identified in atopic asthma in two studies with blood methylation: the Swiss Cohort Study on Air Pollution and Lung and Heart Diseases in Adults (SAPALDIA) and the Pregnancy and Childhood Epigenetics Consortium (PACE) meta-analysis [27]; in four studies with nasal methylation: the Epigenetic Variation and Childhood Asthma in Puerto Ricans (EVA-PR) study [29], the Inner-City Asthma Consortium (ICAC) study [30], the Prevention and Incidence of Asthma and Mite Allergy (PIAMA) study [29] and Project Viva [31]; in one study with eosinophil methylation: the Saguenay–Lac-Saint-Jean (SLSJ) study [32, 33]; and in one study with bronchial epithelium methylation [34]. FDR significant CpG sites identified in non-atopic asthma were looked up in SAPALDIA, the PACE meta-analysis, the EVA-PR study, Project Viva and the SLSJ study.

Results

Table 1 includes descriptive characteristics of the study population. The median age across the four outcome groups ranged from 60–63 years. Participants with atopic asthma were more likely to have adequately controlled asthma than participants with non-atopic asthma (58% *versus* 45%).

Atopy alone

No CpG sites were differentially methylated among individuals with atopy without asthma ($n=185$) compared to non-cases (neither atopy nor asthma) ($n=1157$) at the FWER ($p < 9 \times 10^{-8}$) or a FDR less than 0.05 ($\lambda=0.97$) (supplementary figure E2). Using a lower atopy cut-off (≥ 0.35 IU·mL⁻¹), we compared 528 participants with atopy alone to 814 non-cases and no CpG sites were significant. No additional analyses were conducted.

Non-atopic asthma

For non-atopic asthma participants ($n=673$) compared to non-cases ($n=1157$), eight CpG sites were differentially methylated using the FWER ($p < 9 \times 10^{-8}$) and 524 at a FDR less than 0.05 (figure 1). The top

TABLE 1 Characteristics of participants in the Agricultural Lung Health Study (ALHS) stratified by asthma and atopy status (n=2286)

Characteristic	Non-cases [#] (n=1157)	Atopy alone (n=185)	Non-atopic asthma (n=673)	Atopic asthma (n=271)
Sex				
Male	625 (54.0)	110 (59.5)	300 (44.6)	138 (50.9)
Female	532 (46.0)	75 (40.5)	373 (55.4)	133 (49.1)
Smoking status				
Never	754 (65.2)	121 (65.4)	456 (67.8)	192 (70.9)
Former	342 (29.6)	56 (30.3)	201 (29.9)	68 (25.1)
Current	61 (5.3)	8 (4.3)	16 (2.4)	11 (4.1)
State of residence				
Iowa	839 (72.5)	114 (61.6)	502 (74.6)	179 (66.1)
North Carolina	318 (27.5)	71 (38.4)	171 (25.4)	92 (33.4)
Autumn enrolment				
Yes	219 (18.9)	45 (24.3)	179 (26.6)	75 (27.7)
No	938 (81.1)	140 (75.7)	494 (73.4)	196 (72.3)
Currently farming				
Yes	797 (68.9)	130 (70.3)	440 (65.4)	180 (66.4)
No	360 (31.1)	55 (29.7)	233 (34.6)	91 (33.6)
Asthma control				
Adequately controlled	–	–	305 (45.3)	156 (57.6)
Inadequately controlled	–	–	131 (19.5)	44 (16.2)
Missing data	–	–	237 (35.2)	71 (26.2)
ICS use				
Yes	–	–	278 (41.3)	103 (38.0)
No	–	–	395 (58.7)	168 (62.0)
Age at home visit years	63 (55–72)	60 (55–68)	62 (55–71)	60 (52–68)
Pack-years of smoking[¶]	14.5 (2.3–35)	7.3 (0.9–25)	6 (1.5–19)	7.7 (1.5–21.3)
BMI kg·m⁻²	28.8 (25.6–32.6)	29.0 (25.3–32.5)	30.5 (26.9–35.1)	29.6 (26.1–33.9)
Cell type				
Monocyte	0.08 (0.06–0.10)	0.08 (0.06–0.11)	0.07 (0.05–0.10)	0.08 (0.06–0.10)
Neutrophil	0.56 (0.48–0.63)	0.56 (0.47–0.63)	0.57 (0.48–0.65)	0.57 (0.50–0.65)
Eosinophil	0.01 (0.00–0.03)	0.02 (0.00–0.05)	0.02 (0.00–0.04)	0.02 (0.00–0.04)
Lymphocyte	0.34 (0.27–0.42)	0.34 (0.26–0.40)	0.33 (0.25–0.40)	0.32 (0.25–0.40)

Data are presented as n (%) or median (IQR). ICS: inhaled corticosteroids; BMI: body mass index; IQR: interquartile range. [#]: non-cases are participants with neither asthma nor atopy; [¶]: pack-years among former and current smokers.

30 CpG sites are shown in table 2, while all FDR CpG sites are given in supplementary table E1. In 516 of the 524 differentially methylated CpG sites, methylation was lower in non-atopic asthma participants compared to non-cases. Systematic inflation was minimal ($\lambda=1.14$) (supplementary figure E3). All differentially methylated CpG sites had a unimodal methylation distribution.

Results were not appreciably altered after adjusting for the polygenic risk score of asthma or current farming status (supplementary table E2). Repeating analyses using the seven estimated cell types, instead of the four measured and estimated cell types, resulted in greater inflation ($\lambda=1.66$; 127 CpG sites were significant at the FWER, while 2243 were for a FDR less than 0.05) (supplementary table E2). When individuals were recategorised using the less stringent atopy cut-off (≥ 0.35 IU·mL⁻¹) (483 non-atopic asthma participants *versus* 814 non-cases), the number of associations decreased (nine CpG sites at a FDR less than 0.05) (supplementary table E2).

In case-only analyses (*i.e.* those restricted to non-atopic asthma participants), we calculated adjusted odds ratios (ORs) for the 524 differentially methylated CpG sites, comparing 278 inhaled corticosteroid (ICS) users to 395 non-users and found no significant associations (none had FDR less than 0.05). Adjusted ORs comparing 131 non-atopic asthma participants with inadequately controlled asthma to 305 with controlled asthma identified differential methylation (FDR less than 0.05) by asthma control at 17 of the 524 CpG sites (OR range 0.63–0.95) (supplementary table E3).

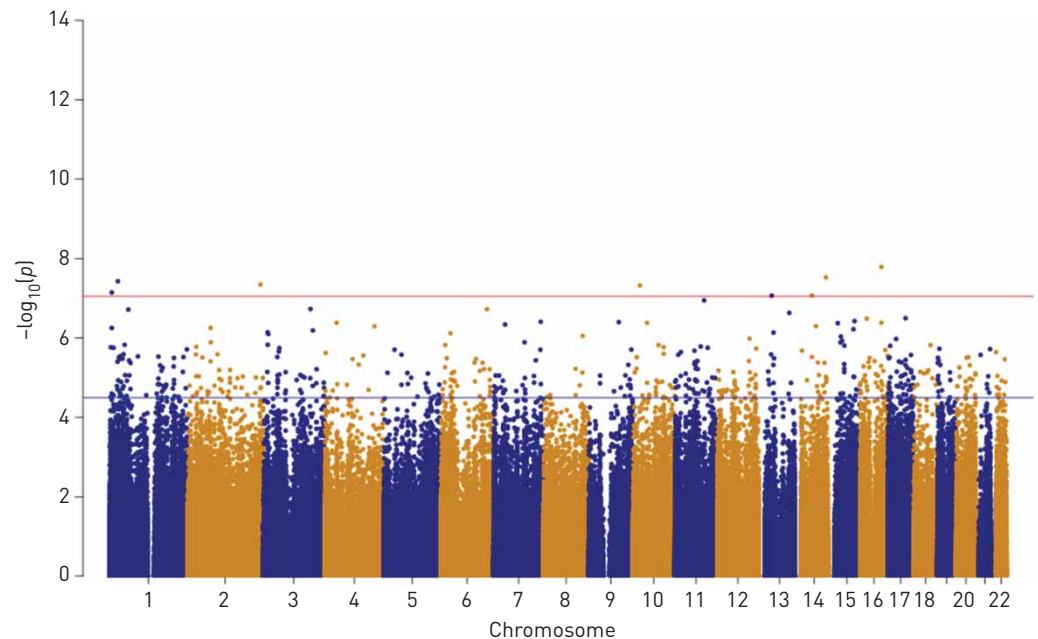


FIGURE 1 Manhattan plot of epigenome-wide analysis of non-atopic asthma. The red line represents the family-wise error rate (FWER) threshold and the blue line represents the false discovery rate (FDR) threshold. Analyses were adjusted for age, sex, body mass index (BMI), smoking status, pack-years of smoking, state of residence, autumn enrolment and four cell types (monocyte, lymphocyte, neutrophil and eosinophil).

Functional enrichment

Removing 15 potentially cross-reactive CpG sites [19] left 509 for downstream analysis. These CpG sites were depleted for CpG islands ($p=5.4 \times 10^{-27}$), CpG island shores ($p=0.0004$) and promoters ($p=1.7 \times 10^{-18}$) (supplementary table E4), enriched for transcription factor binding sites ($p=1.4 \times 10^{-13}$) and enriched (FDR less than 0.05) for two transcription factor motifs (V_FOXO3A_Q1 and V_AIRE_01) (supplementary table E5). Enrichment of DNase I hypersensitivity was observed in blood, fetal lung and lung (supplementary figure E4). Of the 15 chromatin states, we observed enrichment for active transcription start sites in blood, as well as enrichment for enhancers, weak transcription and strong transcription in blood and lung (supplementary figure E4). Among histone marks, we found enrichment for H3K4me1 in blood, fetal lung and lung, for H3K4me3 in blood and fetal lung, and for H3K36me3 in blood (supplementary figure E4).

Pathway analyses

Nine pathways showed enrichment ($p < 0.05$), including Alzheimer's disease, amyotrophic lateral sclerosis, long-term potentiation, vascular smooth muscle contraction and calcium signalling (supplementary table E6 and supplementary figures E5 and E6). Two asthma relevant pathways had p-values between 0.05 and 0.10: asthma ($p=0.08$) and sphingolipid metabolism ($p=0.055$).

cis-eQTM

Of the 509 CpG sites associated with non-atopic asthma, 169 CpG sites were on the Illumina 450K array and 168 were available in BIOS [26]. 120 CpG sites (71.4%) were significantly associated (FDR less than 0.05) with 377 gene expressions in *cis* (supplementary table E7).

Druggable targets

The 509 differentially methylated CpG sites annotated to 479 genes. After excluding genes identified from previous asthma EWAS and GWAS (supplementary table E8), 382 genes were novel (including *AZU1* and *PDE4B*). In ChEMBL [28], *PDE4B* is a target of several approved drugs related to asthma or other respiratory diseases (e.g. Roflumilast, Dyphylline and Theophylline) (supplementary table E9).

Replication

Five replication studies assessed methylation in blood, nasal epithelium, or eosinophils. Replication studies generally had smaller sample sizes and not all stratified by atopy (table 3). Of the 509 differentially methylated CpG sites identified in non-atopic asthma, 169 were on the 450K array and 84 of these (50%)

TABLE 2 Top 30 significant cytosine–phosphate–guanine (CpG) sites in non-atopic asthma

Chromosome	Position [#]	CpG	Gene name [¶]	n	OR*	95% CI	p-value	FDR	Coefficient [§]
16	68804850	cg06085527	–	1829	0.93	0.91–0.95	1.62E-08	7.76E-03	–0.0093
14	100610407	cg14084609	<i>DEGS2</i>	1830	0.82	0.76–0.88	2.99E-08	7.76E-03	–0.0033
1	26091858	cg08028384	<i>SELENON</i>	1829	0.93	0.90–0.95	3.73E-08	7.76E-03	–0.0099
2	234608559	cg10180919	<i>UGT1A6</i>	1829	0.89	0.85–0.93	4.52E-08	7.76E-03	–0.006
10	22743835	cg15737719	<i>LOC100499489</i>	1829	0.76	0.68–0.84	4.75E-08	7.76E-03	–0.0023
1	6341287	cg09249800	<i>GPR153</i>	1828	0.93	0.90–0.95	7.16E-08	8.81E-03	–0.0085
14	55603874	cg23575099	<i>LGALS3</i>	1830	0.81	0.76–0.88	8.46E-08	8.81E-03	–0.0029
13	41631052	cg07908654	<i>WBP4</i>	1830	0.86	0.81–0.91	8.62E-08	8.81E-03	–0.0036
11	93456334	cg23338316	<i>SCARNA9</i>	1828	0.87	0.82–0.91	1.13E-07	1.03E-02	–0.0048
3	152215571	cg07952576	<i>TMEM14EP</i>	1828	0.97	0.96–0.98	1.87E-07	1.31E-02	–0.026
6	149450585	cg09639771	<i>TAB2</i>	1830	0.95	0.93–0.97	1.90E-07	1.31E-02	–0.0128
1	59543930	cg18581916	<i>LINC01358</i>	1830	0.95	0.93–0.97	1.93E-07	1.31E-02	–0.0126
13	97846783	cg23933458	<i>LINC00456</i>	1829	0.96	0.94–0.97	2.33E-07	1.46E-02	–0.0158
17	56269767	cg01955639	<i>EPX</i>	1830	0.88	0.84–0.92	3.18E-07	1.58E-02	–0.0038
16	21831372	cg07611887	<i>RRN3P1</i>	1829	0.88	0.84–0.92	3.26E-07	1.58E-02	–0.0053
15	85186517	cg17194668	<i>WDR73</i>	1830	0.86	0.81–0.91	3.76E-07	1.58E-02	–0.0037
7	150773709	cg06807926	<i>FASTK</i>	1828	0.96	0.94–0.98	3.91E-07	1.58E-02	–0.0141
9	95800911	cg03234093	<i>SUSD3</i>	1829	0.95	0.94–0.97	3.99E-07	1.58E-02	–0.013
4	38110810	cg07456972	<i>TBC1D1</i>	1825	0.81	0.75–0.88	4.13E-07	1.58E-02	–0.0025
16	68804845	cg21468244	–	1827	0.95	0.93–0.97	4.14E-07	1.58E-02	–0.0133
10	45495435	cg01614759	<i>ZNF22</i>	1829	0.87	0.82–0.92	4.17E-07	1.58E-02	–0.0037
15	31505024	cg08884974	<i>LINC02352</i>	1829	0.88	0.83–0.92	4.26E-07	1.58E-02	–0.0043
7	36077237	cg24598141	<i>LOC101928618</i>	1827	0.97	0.95–0.98	4.59E-07	1.63E-02	–0.0175
14	68713369	cg01171954	<i>LOC100996664</i>	1828	0.94	0.92–0.96	5.04E-07	1.66E-02	–0.0102
4	159969044	cg15834151	<i>C4orf45</i>	1830	0.93	0.91–0.96	5.08E-07	1.66E-02	–0.0076
2	74612222	cg17988187	<i>DCTN1-AS1</i>	1830	0.89	0.85–0.93	5.62E-07	1.70E-02	–0.0051
1	6341230	cg21220721	<i>GPR153</i>	1828	0.94	0.92–0.97	5.62E-07	1.70E-02	–0.009
15	81623226	cg25466522	<i>TMC3-AS1</i>	1828	0.82	0.76–0.89	6.02E-07	1.76E-02	–0.0024
3	159847104	cg17991030	<i>IL12A-AS1</i>	1829	0.97	0.95–0.98	6.46E-07	1.82E-02	–0.019
3	14708144	cg15106081	<i>C3orf20</i>	1827	0.76	0.68–0.85	7.20E-07	1.93E-02	–0.0017

OR: odds ratio; CI: confidence interval; FDR: false discovery rate. [#]: genome build GRCh37/hg19; [¶]: annotated using HOMER version 4.10.3 and an associated human genome database (hg19, 5v.10) [35]; ^{*}: logistic regression model adjusted for age, sex, body mass index (BMI), smoking status, pack-years of smoking, state of residence, autumn enrolment and four cell types (monocyte, lymphocyte, neutrophil and eosinophil). ORs indicate the multiplicative change in the odds of having non-atopic asthma in relation to an increase in DNA methylation; [§]: adjusted mean methylation difference based on non-atopic asthma status. The coefficient is from a linear regression model with robust standard error estimation, where methylation is the outcome and non-atopic asthma status is the predictor, adjusting for the same covariates as were used in the logistic regression. A coefficient of –0.01 means that the mean methylation value is 0.01 lower in participants with non-atopic asthma compared to non-cases. Possible methylation values range from zero to one.

were replicated ($p < 0.05$, with the same direction of association) in at least one study (supplementary table E10). Project Viva used the EPIC array, which had an additional 314 CpG sites, of which 56 were replicated (supplementary table E10).

Atopic asthma

Comparing atopic asthma participants ($n = 271$) to non-cases ($n = 1157$), we identified 181 differentially methylated CpG sites using the FWER ($p < 9 \times 10^{-8}$) and 1086 with a FDR less than 0.05 (figure 2). The top 30 CpG sites are shown in table 4 and all FDR CpG sites are given in supplementary table E11). In 99.5% of the 1086 differentially methylated CpG sites, methylation was lower in atopic asthma participants than in non-cases. All differentially methylated CpG sites had a unimodal methylation distribution. Systematic inflation was not observed ($\lambda = 0.98$) (supplementary figure E7). Of the 1086 CpG sites, 104 (9.6%) were FDR-significant in non-atopic asthma (supplementary table E12).

Adjustment for the polygenic risk score of asthma or current farming status did not materially alter the results (supplementary table E2). When we adjusted for the seven estimated cell types, instead of the four cell types, the λ value increased to 1.21 (407 CpG sites were significant using the FWER and 2947 were significant with a FDR less than 0.05, including 1076 of the original 1086 CpG sites) (supplementary table E11). When we classified atopy using the cut-off ≥ 0.35 IU·mL⁻¹ (461 atopic asthma participants *versus* 814 non-cases), the

TABLE 3 Summary of look-up replication

Study	Tissue	Study population	Methylation array	Stratified by atopy?	Asthma cases	Controls	Overlap [#]	Replicated [†]	Replicated and overlap in ALHS asthma	
									Non-atopic	Atopic
Non-atopic asthma										
SAPALDIA	Blood	Adults	450K	Non-atopic	70	134	169 [100]	0 [0]		0
PACE	Blood	Children	450K	Did not stratify	631	2862	168 [99.4]	68 [40.5]		41
meta-analysis										
EVA-PR	Nasal	Children	450K	Non-atopic	66	104	146 [86.4]	8 [5.5]		5
Project Viva	Nasal	Children	EPIC	Did not stratify	65	398	471 [92.5]	143 [30.4]		86
SLSJ	Eosinophil	Children and adults	450K	Did not stratify	16	8	168 [99.4]	49 [29.2]		39
Atopic Asthma										
SAPALDIA	Blood	Adults	450K	Atopic	91	134	349 [100]	0 [0]		0
PACE	Blood	Children	450K	Did not stratify	631	2862	347 [99.4]	288 [83.0]		41
meta-analysis										
EVA-PR	Nasal	Children	450K	Atopic	169	104	299 [83.3]	235 [78.6]		28
ICAC	Nasal	Children	450K	Atopic	36	36	349 [100]	222 [63.6]		26
PIAMA	Nasal	Children	450K	Atopic	27	219	349 [100]	63 [18.1]		16
Project Viva	Nasal	Children	EPIC	Atopic	36	265	1008 [94.2]	875 [86.8]		86
SLSJ	Eosinophil	Children and adults	450K	Did not stratify	16	8	338 [96.8]	274 [81.1]		39
Bronchial epithelium	Airway epithelial	Adults	450K	Did not stratify	74	41	254 [72.8]	49 [19.3]		4

Data are presented as n or n (%). ALHS: Agricultural Lung Health Study; SAPALDIA: Swiss Cohort Study on Air Pollution and Lung and Heart Diseases in Adults; PACE: Pregnancy and Childhood Epigenetics Consortium; EVA-PR: Epigenetic Variation and Childhood Asthma in Puerto Ricans; ICAC: Inner-City Asthma Consortium; PIAMA: Prevention and Incidence of Asthma and Mite Allergy; SLSJ: Saguenay-Lac-Saint-Jean. [#]: overlap with ALHS false discovery rate (FDR) results. For 450K, percentages were based on 169 cytosine-phosphate-guanine (CpG) sites for non-atopic asthma and 349 CpG sites for atopic asthma. For EPIC, percentages were based on 509 CpG sites for non-atopic asthma and 1070 CpG sites for atopic asthma; [†]: CpG sites in the replication study with $p < 0.05$ and the same direction of association as observed in the ALHS. Percentages were based on the number of CpG sites that overlap with the replication study.

number of associations decreased (124 CpG sites were significant using the FWER and 847 were significant with a FDR less than 0.05, including 669 of the original 1086 CpG sites) (supplementary table E2).

Conducting adjusted logistic regression analyses restricted to participants with atopic asthma, none of the 1086 significant CpG sites were differentially methylated (all FDR greater than 0.05) in relation to ICS use (103 users *versus* 168 non-users) or asthma control (44 with inadequately controlled asthma *versus* 156 with adequately controlled asthma).

Functional enrichment

The removal of 16 potentially cross-reactive probes [19] left 1070 differentially methylated CpG sites for downstream analysis. We found depletion for CpG islands ($p = 4.3 \times 10^{-66}$), CpG island shores ($p = 1.3 \times 10^{-6}$) and promoters ($p = 1.0 \times 10^{-46}$), enrichment for transcription factor binding sites ($p = 0.0057$) (supplementary table E4) and enrichment for eight transcription factor motifs (including GATA3_{primary} and MA0029.1-Evi1) (supplementary table E5). Among the 15 chromatin states, we identified enrichment of enhancers, genic enhancers, weak transcription and strong transcription in blood and lung (supplementary figure E8). The FDR significant CpG sites were enriched for H3K4me1 and H3K36me3 in blood, fetal lung and lung (supplementary figure E8).

Pathway analyses

Ten KEGG pathways had $p < 0.05$, including insulin signalling, type II diabetes, starch and sucrose metabolism, and valine, leucine, and isoleucine degradation (supplementary table E6 and supplementary figures E5 and E6). The asthma pathway was marginally significant ($p = 0.055$). Figure 3 shows a network plot of nine disease and biological pathways implicated by genes identified in our non-atopic and atopic asthma analyses.

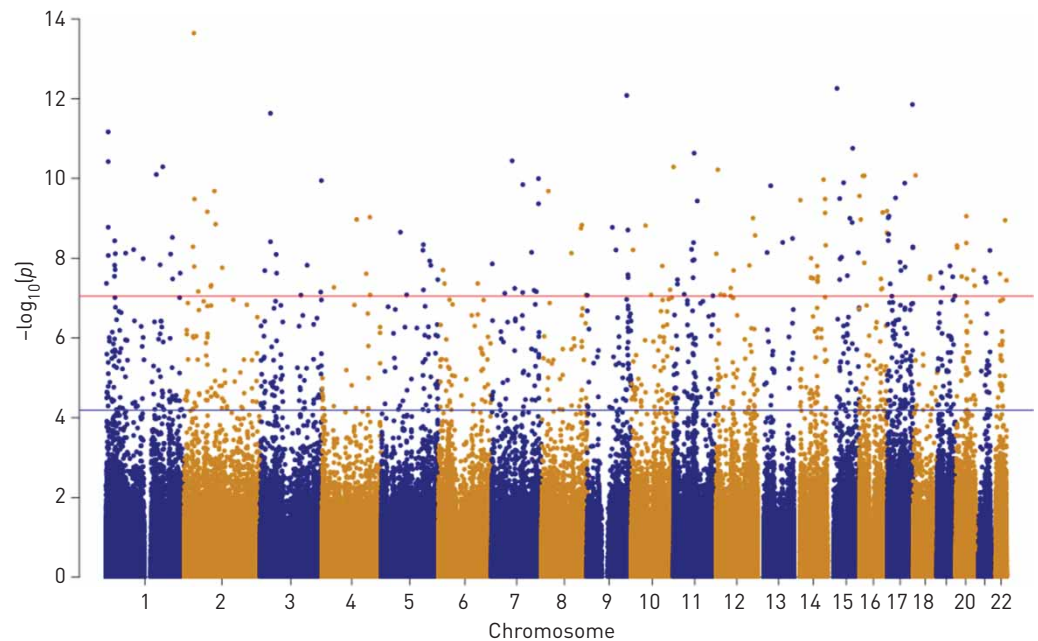


FIGURE 2 Manhattan plot of epigenome-wide analysis of atopic asthma. The red line represents the family-wise error rate (FWER) threshold and the blue line represents the false discovery rate (FDR) threshold. Analyses were adjusted for age, sex, body mass index (BMI), smoking status, pack-years of smoking, state of residence, autumn enrolment and four cell types (monocyte, lymphocyte, neutrophil and eosinophil).

cis-eQTM

Of the 1070 CpG sites associated with atopic asthma, 349 were present in the 450K array and 346 were available in BIOS. 242 CpG sites (69.9%) were significantly associated with 671 gene expressions in *cis* (supplementary table E13).

Druggable targets

The 1070 CpG sites annotated to 885 genes, of which 569 have not been reported in previous asthma EWAS or GWAS (including *PDE4B* and *PPARG*). In ChEMBL [28], *PPARG* is a target of a candidate asthma drug (Rosiglitazone) [36, 37] (supplementary table E14).

Replication

Eight replication studies assessed methylation in blood, nasal epithelium, bronchial epithelium, or eosinophils (table 3). The 1076 CpG sites identified in atopic asthma included 349 on the 450K array and 341 of these (98%) were replicated in at least one study (supplementary table E15). An additional 684 CpG sites were available in Project Viva, which assessed nasal methylation using the EPIC array and 595 of these (87%) were replicated (supplementary table E15).

Discussion

Several hundred CpG sites in blood were differentially methylated in blood DNA from adults with non-atopic or atopic asthma compared to adults with neither asthma nor atopy. Many more CpG sites were differentially methylated in relation to atopic rather than non-atopic asthma and about 10% of those for atopic asthma overlapped with non-atopic asthma. Our findings highlight the importance of distinguishing between non-atopic and atopic types to improve our understanding of asthma and show that differential methylation in atopic asthma is not driven by atopy *per se*.

We performed several analyses to further evaluate our findings. Results were similar after adjustment for the polygenic risk score of asthma, suggesting that our findings are not influenced by known genetic variations of asthma. In most participants, we directly measured differential blood counts for monocytes, lymphocytes, neutrophils and eosinophils. Adjustment for all seven estimated cell types instead, which disaggregates lymphocytes into NK, B-cell, CD4⁺ and CD8⁺ types, resulted in some inflation; however, results were similar. Eosinophils are higher in individuals with asthma than the general population [38]. Estimation of eosinophils from a small reference panel of healthy participants may not be highly accurate, as suggested in our data (Spearman correlation between measured and estimated=0.09). Using a less stringent atopy cut-off

TABLE 4 Top 30 significant cytosine–phosphate–guanine (CpG) sites in atopic asthma

Chromosome	Position [#]	CpG	Gene name [¶]	n	OR [*]	95% CI	p-value	FDR	Coefficient [§]
2	31302644	cg26382374	<i>GALNT14</i>	1425	0.76	0.71–0.82	2.27E-14	1.86E-08	−0.0101
15	31248701	cg16606719	<i>MTMR10</i>	1428	0.72	0.66–0.79	5.51E-13	2.25E-07	−0.0066
9	126497597	cg01745810	<i>DENND1A</i>	1428	0.76	0.71–0.82	8.29E-13	2.26E-07	−0.0082
17	79851485	cg12380988	<i>ANAPC11</i>	1428	0.74	0.68–0.80	1.40E-12	2.86E-07	−0.0075
3	33112660	cg26396322	<i>TMPPE</i>	1428	0.72	0.66–0.79	2.32E-12	3.79E-07	−0.006
1	6341287	cg09249800	<i>GPR153</i>	1428	0.88	0.85–0.91	6.80E-12	9.26E-07	−0.0161
15	81623226	cg25466522	<i>TMC3-AS1</i>	1426	0.70	0.63–0.78	1.75E-11	2.04E-06	−0.0051
11	65546210	cg05300717	<i>AP5B1</i>	1421	0.87	0.84–0.91	2.32E-11	2.37E-06	−0.0121
7	65439512	cg04290133	<i>GUSB</i>	1422	0.81	0.75–0.86	3.61E-11	3.09E-06	−0.008
1	6341327	cg11699125	<i>GPR153</i>	1426	0.90	0.87–0.93	3.78E-11	3.09E-06	−0.0191
1	180940378	cg11649969	<i>STX6</i>	1428	0.60	0.51–0.70	5.10E-11	3.49E-06	−0.0032
10	135061670	cg12227660	<i>MIR202HG</i>	1419	0.88	0.84–0.91	5.13E-11	3.49E-06	−0.0158
12	6342778	cg20560376	<i>CD9</i>	1427	0.71	0.64–0.79	6.03E-11	3.79E-06	−0.0056
1	160309220	cg09332506	<i>NCSTN</i>	1428	0.53	0.44–0.64	7.96E-11	4.15E-06	−0.0027
18	8720395	cg27550672	<i>MTCL1</i>	1428	0.68	0.61–0.76	8.37E-11	4.15E-06	−0.0038
16	16143033	cg24304533	<i>ABCC1</i>	1426	0.73	0.67–0.81	8.52E-11	4.15E-06	−0.0057
16	11709855	cg24517604	<i>LITAF</i>	1425	0.77	0.71–0.83	8.64E-11	4.15E-06	−0.0068
7	149543136	cg05184016	<i>ZNF862</i>	1427	0.68	0.61–0.76	1.01E-10	4.59E-06	−0.0048
14	95615731	cg01901579	<i>DICER1</i>	1428	0.78	0.72–0.84	1.07E-10	4.60E-06	−0.0074
3	195974300	cg02803925	<i>SLC51A</i>	1424	0.77	0.71–0.83	1.13E-10	4.62E-06	−0.0051
15	52427175	cg05853552	<i>BCL2L10</i>	1428	0.74	0.67–0.81	1.28E-10	4.87E-06	−0.0063
17	55190679	cg13947225	<i>AKAP1</i>	1424	0.81	0.76–0.87	1.31E-10	4.87E-06	−0.0083
7	99507261	cg17717565	<i>TRIM4</i>	1421	0.84	0.79–0.88	1.43E-10	5.08E-06	−0.0103
13	41631052	cg07908654	<i>WBP4</i>	1428	0.78	0.73–0.84	1.53E-10	5.21E-06	−0.0068
2	96965099	cg01923915	<i>SNRNP200</i>	1428	0.73	0.67–0.81	2.07E-10	6.54E-06	−0.005
8	22169041	cg16427256	<i>PIWIL2</i>	1428	0.76	0.69–0.82	2.08E-10	6.54E-06	−0.0052
16	616212	cg04497992	<i>NHLRC4</i>	1428	0.87	0.83–0.91	2.75E-10	8.32E-06	−0.0127
17	25897247	cg06127160	<i>LGALS9</i>	1428	0.87	0.83–0.91	3.08E-10	8.65E-06	−0.0114
15	40093898	cg18852698	<i>LOC105370941</i>	1427	0.82	0.77–0.87	3.20E-10	8.65E-06	−0.007
2	32946576	cg18399629	<i>MIR4765</i>	1428	0.69	0.62–0.78	3.28E-10	8.65E-06	−0.004

OR: odds ratio; CI: confidence interval; FDR: false discovery rate. [#]: genome build GRCh37/hg19; [¶]: annotated using HOMER version 4.10.3 and an associated human genome database (hg19, 5v.10) [35]; ^{*}: logistic regression model adjusted for age, sex, body mass index (BMI), smoking status, pack-years of smoking, state of residence, autumn enrolment and four cell types (monocyte, lymphocyte, neutrophil and eosinophil). ORs indicate the multiplicative change in the odds of having atopic asthma in relation to an increase in DNA methylation; [§]: adjusted mean methylation difference based on atopic asthma status. The coefficient is from a linear regression model with robust standard error estimation, where methylation is the outcome and atopic asthma status is the predictor, adjusting for the same covariates as were used in the logistic regression. A coefficient of −0.01 means that the mean methylation value is 0.01 lower in participants with atopic asthma compared to non-cases. Possible methylation values range from zero to one.

(≥ 0.35 IU·mL^{−1}) yielded fewer significant findings, consistent with previous evidence that the lower threshold is less discriminatory [7]. Notably, results for atopic asthma were more robust in response to the threshold change (more CpG sites remained significant) than the results for non-atopic asthma.

A substantial proportion of our differentially methylated CpG sites were replicated in at least one published study. A higher proportion were replicated in atopic asthma than in non-atopic asthma, probably reflecting the much larger quantity of studies available for atopic asthma and thus the higher power. Most CpG sites that were replicated in non-atopic asthma overlapped with our findings in atopic asthma (table 3), suggesting that these are asthma-related CpG sites independent of atopy status. Our results in atopic asthma were highly consistent in a small cohort of participants with methylation measured in isolated eosinophils, a critical cell type for atopic asthma. Nasal epithelium is a good surrogate for lower respiratory epithelium [39] and 89% of our findings in atopic asthma were replicated in nasal tissue, suggesting that methylation in blood is a good surrogate for nasal tissue in methylation studies of atopic asthma. This is an important observation, given the wide availability of stored blood for methylation analyses.

In our study and in most of the replication studies, methylation at significant CpG sites tended to be lower in participants with asthma than in individuals without asthma. We hypothesise that lower methylation in individuals with asthma reflects the combination of a cell-type composition which displays lower methylation at these CpG sites [40] and the activation of cell types related to disease processes. For example, there might be a shift from naïve T-cells to effector memory CD8T cells and NK cells [40], or activation of

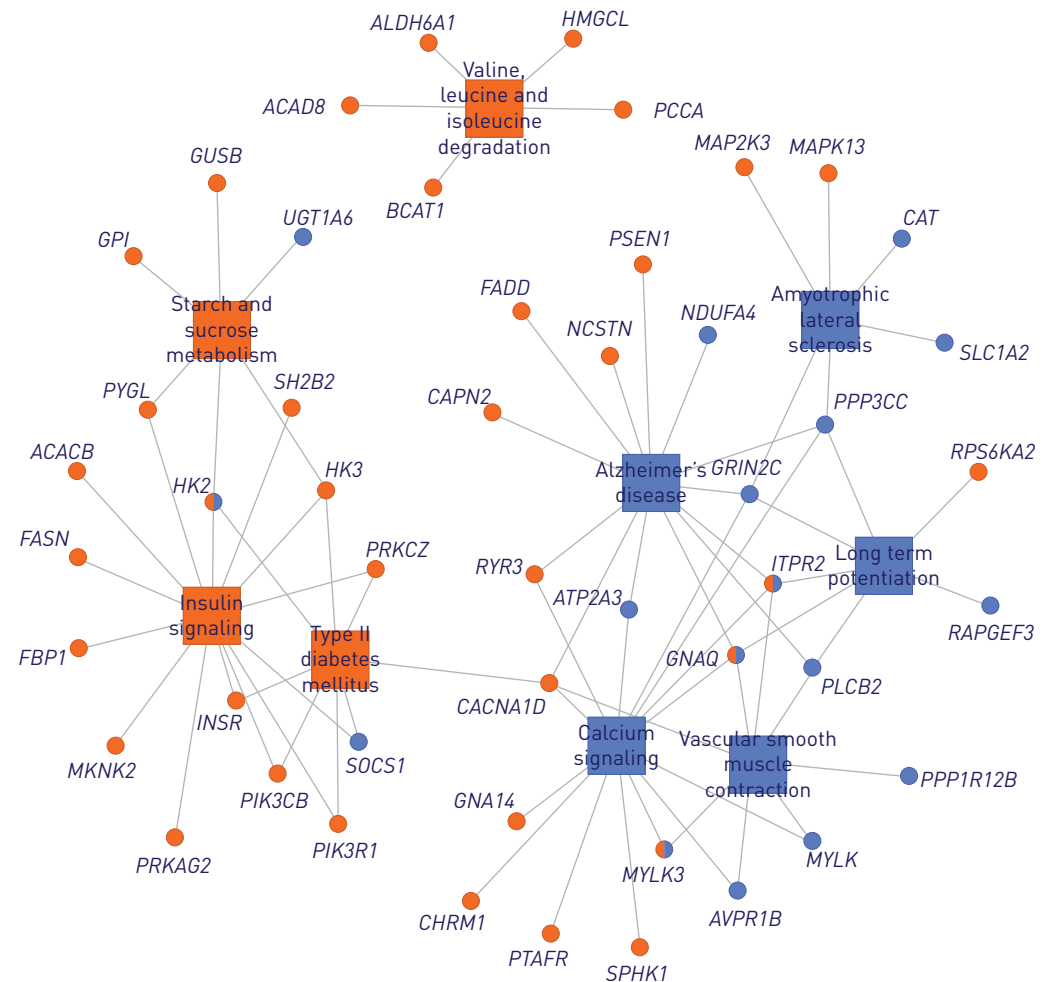


FIGURE 3 Network plot of nine enriched pathways (squares) with connecting implicated genes (circles) from non-atopic and/or atopic asthma. Blue represents genes or pathways implicated in non-atopic asthma. Orange represents genes or pathways implicated in atopic asthma.

basal cells [41]. Mechanistic studies are needed to investigate why methylation is generally lower among individuals with asthma than those without asthma at differentially methylated CpG sites across the studied tissues.

This study provides substantial replication of prior studies. Our analysis of non-atopic asthma participants implicated 479 genes, including 96 identified in previous asthma GWAS or EWAS (supplementary table E16). Of 885 genes implicated in our analysis of atopic asthma, 315 were identified in other GWAS or EWAS of asthma (supplementary table E17). We also identified many novel asthma-related genes not previously implicated in GWAS or EWAS. For example, high expression of *AZU1*, an inflammatory mediator that regulates neutrophils, is correlated with poor asthma control [42]. Additionally, *DEGS2* is involved in sphingolipid metabolism, which has been implicated in respiratory outcomes [43] including asthma [40].

Enrichment of functional genomic features and pathway analyses provide biological insight into our differentially methylated CpG sites. Our results highlighted certain genomic features, histone marks in blood, lung and fetal lung, and transcription factor motifs that might be biologically relevant to asthma. For example, transcription factors *FOXO3A* and *GATA3* help regulate type-2 helper T-cells [44, 45], an important cell type in asthma and *EVI-1* interacts with *SMAD3* [46], a known asthma gene. The differentially methylated CpG sites in non-atopic asthma were enriched in signalling pathways related to the nervous system (e.g. sphingolipid metabolism, calcium signalling and vascular smooth muscle contraction). Sphingolipids and calcium are signalling molecules involved in vascular smooth muscle contraction [47–50], suggesting that mechanisms related to innervation of the airways may play a greater role in non-atopic asthma *versus* atopic asthma. Atopic asthma CpG sites were enriched in pathways involved in inflammatory response (i.e. insulin signalling) [51] or characterised by chronic inflammation (i.e. diabetes) [52], consistent with the inflammatory nature of atopic asthma.

Previous studies have identified CpG sites differentially methylated in relation to total serum IgE [33, 53–55], atopy [29, 56, 57] and allergies [56, 58] but, in our study, no CpG sites were differentially methylated in relation to atopy alone. As previous studies have been conducted in children, or a combination of children and adults, and our study is comprised of older adults (median age 62 years), it is possible that differential methylation related to atopy in children may not persist into adulthood.

This study has some limitations. Our population was rural and may differ in environmental factors compared to urban populations (e.g. air pollution). However, we found high levels of replication across geographically distinct populations from published studies. Furthermore, heterogeneity in disease phenotypes likely remains even though we stratified asthma by atopy status and, as the study is cross-sectional, we cannot determine if findings reflect the pathogenesis or consequences of asthma.

This study also has several strengths. It is the largest EWAS of adult asthma that has been conducted and the substantial replication in nasal epithelium and purified eosinophils suggests that methylation in blood is a good proxy for asthma-relevant tissues in discovering novel differential methylation in atopic asthma. Pathway analyses support the biological plausibility of our findings and the CpG sites implicated in blood are enriched for localisation to functional genomic elements in blood and lung. Many CpG sites were associated with gene expression in both non-atopic and atopic asthma, further supporting the potential functional impact of the differential methylation we identified at these loci. Different findings for non-atopic and atopic asthma confirm the importance of separating forms of asthma to discover novel associations, which may better inform disease aetiology and lead to improved treatment strategies.

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